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Effect of pH and added sugar on stability of color, anthocyanin content and phenolic content of *Clitoria ternatea*, *Ipomoea tricolor* and *Brassica oleracea* extracts

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Abstract

The effect was determined of different types of sugar (sucrose, glucose, maltose) at different concentrations (10%, 20%, 30%) on the stability of three sources of anthocyanin extract (*Clitoria ternatea* flowers, *Ipomoea tricolor* flowers, *Brassica oleracea* leaves) during storage at pH 5 and pH 6. The anthocyanin of plant samples was extracted using deionized water for 30 min at 60°C with continuous shaking. The stability parameters in the study were color intensity, total anthocyanin content and total phenolic content. The highest stability was recorded for *C. ternatea*, followed by *I. tricolor* and *B. oleracea*, respectively. The half-life vales of color intensity of *C. ternatea* at pH 5 and pH 6 with various sugar concentrations added were in the range 77.88–231.05 d, while the half-life values of *Ipomoea tricolor* and *Brassica oleracea* were 37.27–87.74 d and 23.03–48.81 d, respectively. Increasing the amount of added sugar increased the stability of all the extracts. Compared to the extracts without sugar, the addition of 30% sugar increased the half-lives of color intensity, total anthocyanin content and total phenolic content for all extracts by 1.54–2.01 times, 1.90–2.26 times and 1.76–2.11 times, respectively. However, the type of sugar had no significant effect on any of the stability parameters of the extracts.

Introduction

Anthocyanins are one of the most important natural food colorants because of their bright, attractive colors and water solubility, which allows their incorporation into aqueous food systems (Bakowska-Barczak, 2005). There are more than 900 types of anthocyanin that can be categorized as unacylated, monoacylated and polyacylated (Yoshida et al., 2009). They provide a wide range of colors, from red to blue depending on their chemical structure and pH. However, the unacylated and monoacylated anthocyanins rapidly lose their color in the pH range of most food (low-acidic to neutral conditions). Hence, they are considered as poor food colorants.

The polyacylated anthocyanins have been reported to have much higher color stability, due to their natural protection against hydration by water (Bakowska-Barczak, 2005). Marpaung et al. (2017) reported that color fading of an anthocyanin is initiated by the hydration of the red species (flavylium cation) to the colorless species (hemiketal). In a polyacylated anthocyanin, the hydration is blocked by the intramolecular co-pigmentation between two aromatic acids and the anthocyanin chromophore (Marpaung et al., 2017).

Butterfly pea (*Clitoria ternatea*) flowers, morning glory (*Ipomoea tricolor*) flowers and red cabbage (*Brassica oleracea*) leaves are three sources of polyacylated anthocyanins (Fig. 1). The fully opened

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butterfly pea flower contains nine types of polyacylated anthocyanins called ternatins (Kazuma et al., 2003). The most complex ternatin is ternatin A1 that contains four aromatic acid moieties (Terahara et al., 1990). The morning glory flower contain one polyacylated anthocyanin known as heavenly blue anthocyanin (HBA). Red cabbage leaves contain three polyacylated anthocyanins with each two acyl groups (Yoshida et al., 2009). While these are much more stable than the most sources of anthocyanin extracts at pH 4 to 7, their color stability is much lower than that required for an artificial food colorant. Therefore, color stability improvement of the polyacylated anthocyanins is needed.

Sugar addition has been reported to affect anthocyanin stability, depending on the sugar concentration, with sufficient quantity affecting the water activity and resulting in a protective effect, while in much smaller quantities, it can accelerate anthocyanin degradation (Delgado-Vargas et al., 2000). However, in contrast, Chu et al. (2016) reported that sugar had no influence on or even tended to antagonize anthocyanin stability. Furthermore, different types of sugar may also have different effects on the stability of anthocyanins (Nikkhah et al., 2007).

The current work aimed to study the effect of different types and concentrations of sugar on the stability of anthocyanin extracts sourced from butterfly pea flowers, morning glory flowers and red cabbage leaves at pH 5 and pH 6. These pH levels were chosen as representative of the pH range in food systems.

Materials and Methods

Materials

Flowers of butterfly pea—*Clitoria ternatea* (CT)—and morning glory—*Ipomoea tricolor* (IT)— were harvested from a garden in South Tangerang, Banten, Indonesia. Leaves of red cabbage—*Brassica oleracea* (BO) —were obtained from a local market (Tangerang, Banten, Indonesia). The petals from CT and IT were separated from the calyx, while the BO was cut into small slices (≤ 4 cm^2). All plant samples were steam-blanched for 6 min (Marpaung et al., 2013), then dried at 45°C for 24 h. The dried samples were pulverized and sieved through a 250 µm screen, then packed in an airtight container and kept in a freezer until used. Deionized water (Amidis®) was obtained from the local market (Tangerang, Banten, Indonesia). Buffer solutions of pH 5 and pH 6 (citric acid-sodium hydroxide), HCl 1 M, sodium carbonate, Folin-Ciocalteu reagent, gallic acid, sucrose, glucose and maltose were obtained from Certipur® (Merck KGaA, Darmstadt, Germany). All reagents were analytical grade and used without further purification.

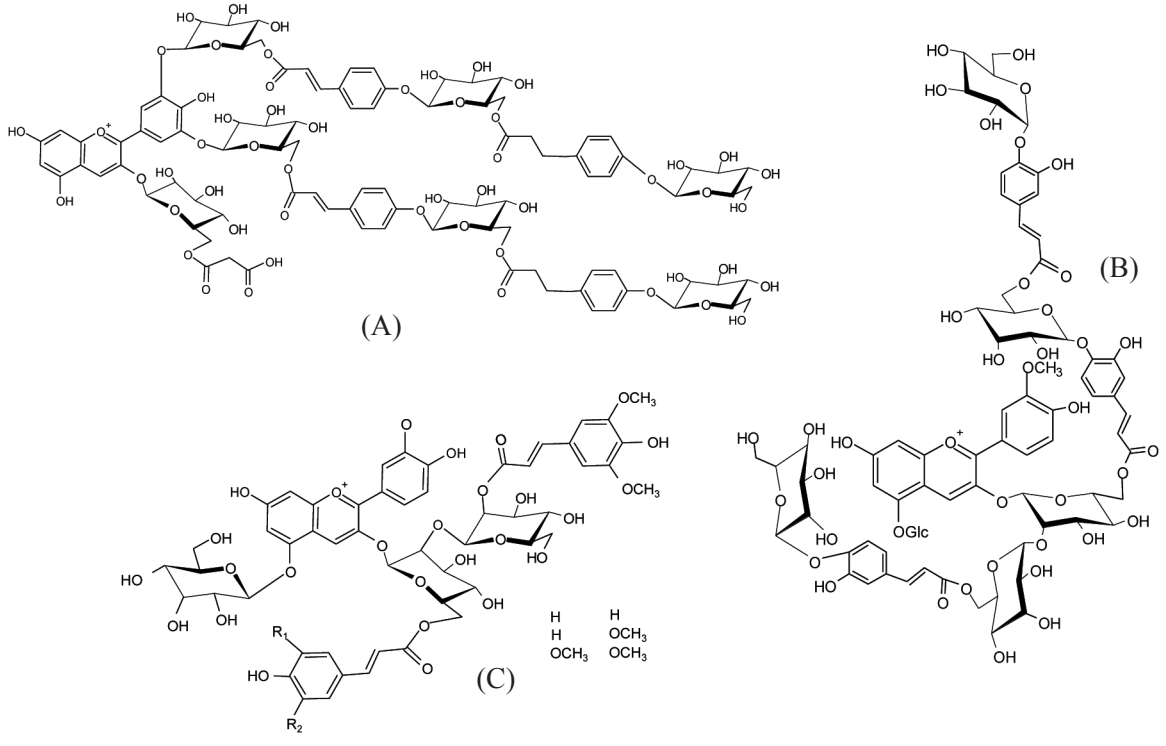

Fig. 1 Chemical structures of (A) ternatin A1, the largest anthocyanin in *Clitoria ternatea* flowers; (B) heavenly blue anthocyanin in *Ipomoea tricolor* flowers; (C) diacylated anthocyanin in *Brassica oleracea* leaves

Extraction

The extraction of anthocyanin from the samples was based on the procedure of Marpaung et al. (2013) with modification. Each sample (1 g) was added to 40 mL of deionized water and extracted at 60°C for 30 min with continuous shaking and no light exposure. The suspensions were filtered through Whatman #41 filter paper.

The filtered extract was adjusted using a buffer solution of pH 5 or pH 6 by a dilution factor of 10, then added with 10%, 20% or 30% sugar (sucrose, glucose, maltose).

Stability test

All 54 treatments were packed in glass containers covered with aluminum foil and stored for 21 d at room temperature. The remaining color intensity (CI), total anthocyanin (TA) and phenolic content (PC) after storage were spectrophotometrically determined using a UV-Vis spectrophotometer (Genesys 10uv; Thermo Electron Corporation; Madison, WI, USA) every 3 d. CI was determined using Equation 1:

$$CI = (A_{\lambda max} - A_{700}) \times DF \quad (1)$$

where $A_{\lambda max}$ is the absorbance at λ_{max} in the visible region, A_{700} is the absorbance at 700 nm and DF is the dilution factor.

The TA was calculated based on the single pH method using cyanidin 3-glucoside (Aishah et al., 2013). A 1.5 mL extract was adjusted with 1 M HCl to reach pH 1, and the absorbance at λ_{max} was measured using Equation 2 and Equation 3:

$$A = (A_{\lambda max} - A_{700}) \quad (2)$$

$$TA = (A \times MW \times DF \times 1{,}000) / (\varepsilon \times l) \quad (3)$$

where MW is molecular weight of cyanidin-3-glucoside (449.2 g/mol), DF is the dilution factor, ε is the molar absorptivity of cyanidin-3glucoside (26,900) and l is the cuvette width.

The PC was determined using the Folin-Ciocalteu method with modification (Cavalcanti et al., 2011). The gallic acid standard was made by diluting 1,000 ppm gallic acid solution to 50 mg/L gallic acid, 100 mg/L gallic acid, 125 mg/L gallic acid, 150 mg/L gallic acid and 200 mg/L gallic acid; then, the absorbance was measured at 765 nm.

The PC was analyzed by placing 0.1 mL of each sample into two different test tubes and then adding 0.5 mL of Folin-Ciocalteu reagent to each test tube and vortex mixing. An amount of 0.4 mL sodium carbonate was added to each test tube and mixed again using vortexing. The mixture was incubated for 60 min in the dark. After incubation, the mixture was placed into different cuvettes and the absorbance was measured using the spectrophotometer at 765 nm. The PC was stated as gallic acid equivalent (GAE) using Equation 4:

$$PC\ (mg/L\ GAE) = A / m \quad (4)$$

where A is the absorbance, and m is the slope of the gallic acid standard curve.

Degradation kinetics

The degradation kinetics of color intensity, total anthocyanin and total phenolic content of all extracts were evaluated based on a first order reaction as shown in Equation 5:

$$A = A_o.e^{-kt} \quad (5)$$

where A is the final concentration, A_o is the initial concentration, e is the base for natural logarithms, k is the constant degradation rate per day and t is the storage time in days.

Statistical analyses

The statistical analyses involved in this research were three way analysis of variance (Design Expert® 7.0 software; Stat-Ease, Inc.; Minneapolis, MN, USA) and trend analyses based on Pearson's correlation and regression analysis (Microsoft Excel® 2010 software; Microsoft Corporation; Redmond, WA, USA). Significance in all analyses was tested at $\alpha = 0.05$. Graph was generated using Design Expert® 7.0 and modified using the Microsoft Powerpoint® software (Microsoft Corporation; Redmond, WA, USA).

Results and Discussion

The color loss of an anthocyanin can be reversible or irreversible, where reversible loss occurs when the flavylium cation is hydrated to colorless hemiketal, whereas irreversible color fading occurs when the anthocyanin degrades to 4-hydroxybenzoic acid and benzaldehyde and in general, at pH 3 or higher, reversible color loss precedes irreversible loss (Marpaung et al. 2017).

Anthocyanin decrease occurs when the anthocyanin is irreversibly deglycosylated to anthocyanidin and finally degraded to 4-hydroxybenzoic acid and a benzaldehyde derivative (Marpaung et al., 2017). In most anthocyanin at pH ≥ 3, anthocyanin degradation is much slower than color fading so commonly, there is a relatively high anthocyanin content in a pale extract (Trouillas et al., 2016).

The PC was used to estimate the total amount of phenolic compounds in the extract including anthocyanins, flavonoids and non-flavonoids phenolic compounds.

The first-order reaction model was adequate to describe the kinetic degradation of CI, TA and PC in all extracts ($p < 0.05$ for regression analyses), except for the anthocyanin degradation of CT extract at pH 5. The degradation rate (k) and half-life ($t_{0.5}$) of CI, TA and PC are summarized in Table 1.

Effect of pH

In general, the color, anthocyanin, and phenolic compound of all extracts studied were more stable at pH 5 than at pH 6. This performance was in accordance with the common characteristics of anthocyanins of decreased stability as the pH increases (Tsai et al., 2004). Under low acidic conditions, anthocyanins were present in the three colorless species (hemiketal, cis- and trans-chalcone) and three colored species (red flavylium cation, purple quinonoidal base, blue quinonoidal base). As the pH increases, the equilibrium shifts to the deprotonation of the flavylium cation to the quinonoidal base that is less stable (Trouillas et al., 2016; Yoshida et al., 2009). Hence, the anthocyanin is less stable at the higher pH. As the anthocyanin degraded, the color faded and the total phenolic compound decreased.

Table 1 Degradation rate and half-life of color intensity (CI), total anthocyanin content (TA) and total phenolic content (TP) of extracts of *Clitoria ternatea* flowers (CT), *Ipomoea tricolor* flowers (IT) and *Brassica oleracea* leaves (BO) at pH 5-6 with addition of various sugar types and concentrations

Source	pH	Sugar type	Concentration (%)	Degradation rate (per day)			Half-life (per day)[1]		
				CI	TA	TP	CI	TA	TP
CT	5	Sucrose	10	0.0040	n.d	0.0076	173.29	n.d	91.20
			20	0.0037	n.d	0.0062	187.34	n.d	111.80
			30	0.0030	n.d	0.0048	231.05	n.d	144.41
		Glucose	10	0.0039	n.d	0.0074	177.73	n.d	93.67
			20	0.0037	n.d	0.0060	187.34	n.d	115.52
			30	0.0031	n.d	0.0047	223.60	n.d	147.48
		Maltose	10	0.0041	n.d	0.0071	169.06	n.d	97.63
			20	0.0038	n.d	0.0067	182.41	n.d	103.45
			30	0.0032	n.d	0.0046	216.61	n.d	150.68
		Control		0.0059	n.d	0.0099	117.48	n.d	70.01
	6	Sucrose	10	0.0074	0.0107	0.0082	93.67	54.58	84.53
			20	0.0064	0.0077	0.0075	108.30	90.02	92.42
			30	0.0053	0.0059	0.0059	130.78	117.48	117.48
		Glucose	10	0.0076	0.0108	0.0089	91.20	54.15	77.88
			20	0.0062	0.0071	0.0074	111.80	97.63	93.67
			30	0.0051	0.0058	0.0060	135.91	119.51	115.52
		Maltose	10	0.0073	0.0107	0.0085	94.95	54.58	81.55
			20	0.0065	0.0071	0.0073	106.64	97.63	94.95
			30	0.0053	0.0054	0.0061	130.78	128.36	113.63
		Control		0.0089	0.0125	0.0106	77.88	55.45	65.39
IT	5	Sucrose	10	0.01250	0.0057	0.0092	55.45	121.60	75.34
			20	0.01050	0.0047	0.0075	66.01	147.48	92.42
			30	0.00800	0.0040	0.0056	86.64	173.29	123.78
		Glucose	10	0.01260	0.0059	0.0089	55.01	117.48	77.88
			20	0.01070	0.0045	0.0079	64.78	154.03	87.74
			30	0.00790	0.0037	0.0054	87.74	187.34	128.36
		Maltose	10	0.01240	0.0060	0.0093	55.90	115.52	74.53
			20	0.01030	0.0049	0.0079	67.30	141.46	87.74
			30	0.00810	0.0040	0.0053	85.57	173.29	130.78
		Control		0.01420	0.0074	0.0104	48.81	93.67	66.65
	6	Sucrose	10	0.0134	0.0090	0.0092	51.73	77.02	75.34
			20	0.0128	0.0061	0.0075	54.15	113.63	92.42
			30	0.0101	0.0051	0.0056	68.63	135.91	123.78
		Glucose	10	0.0136	0.0091	0.0089	50.97	76.17	77.88
			20	0.0129	0.0066	0.0079	53.73	105.02	87.74
			30	0.0103	0.0050	0.0054	67.30	138.63	128.36
		Maltose	10	0.0135	0.0093	0.0093	51.34	74.53	74.53
			20	0.0128	0.0068	0.0079	54.15	101.93	87.74
			30	0.0100	0.0053	0.0053	69.31	130.78	130.78
		Control		0.0186	0.0107	0.0104	37.27	64.78	66.65
BO	5	Sucrose	10	0.0225	0.0350	0.0092	30.81	19.80	75.34
			20	0.0182	0.0263	0.0075	38.09	26.36	92.42
			30	0.0142	0.0200	0.0056	48.81	34.66	123.78
		Glucose	10	0.0227	0.0337	0.0089	30.54	20.57	77.88
			20	0.0184	0.0244	0.0079	37.67	28.41	87.74
			30	0.0147	0.0171	0.0054	47.15	40.53	128.36
		Maltose	10	0.0224	0.0355	0.0093	30.94	19.53	74.53
			20	0.0180	0.0237	0.0079	38.51	29.25	87.74
			30	0.0143	0.0188	0.0053	48.47	36.87	130.78
		Control		0.0289	0.0420	0.0104	23.98	16.50	66.65
	6	Sucrose	10	0.0248	0.0354	0.0122	27.95	19.58	56.82
			20	0.0234	0.0289	0.0094	29.62	23.98	73.74
			30	0.0194	0.0201	0.0079	35.73	34.48	87.74
		Glucose	10	0.0249	0.0332	0.0125	27.84	20.88	55.45
			20	0.0233	0.0274	0.0095	29.75	25.30	72.96
			30	0.0195	0.0205	0.0082	35.55	33.81	84.53
		Maltose	10	0.0245	0.0337	0.0121	28.29	20.57	57.28
			20	0.0235	0.0270	0.0097	29.50	25.67	71.46
			30	0.0196	0.0217	0.0083	35.36	31.94	83.51
		Control		0.0301	0.0414	0.0143	23.03	16.74	48.47

Interestingly, despite the color of the CT extract at pH 5 decreasing at a rate of 0.0038% of the initial color intensity per day, the total anthocyanin remained stable, as modelled in Fig. 2. This result indicated that the color degradation of the CT extract at pH 5 was in the reversible stage. A similar phenomenon was also exhibited by the IT extract, with the anthocyanin degrading more slowly than the color fading. In contrast, the anthocyanin in the BO extract degraded more rapidly than the color. This could be best explained by the anthocyanin degrading while the color intensity was maintained slightly higher because of the self-association among anthocyanins as also happens in red wine (Boulton, 2001). However, further study is needed to confirm the proposed explanation.

Effect of sugar

Sugar addition is considered to improve the color stability of anthocyanin source extracts by binding with water molecules and therefore, their availability to hydrate the flavylium cation is limited (Chu et al., 2016; Nikkhah et al., 2007). Significant improvement in color stability due to sugar addition has been reported (Tsai et al.,

2004; Chu et al., 2016; Nikkhah et al., 2007)) and a similar result was also evident in the current work as the color stability of all extracts improved as the amount of sugar increased (Fig. 3). However, any effect of the sugar type on color stability was not evident.

The amount of sugar added also significantly increased the anthocyanin stability of all extracts. Hence, sugar addition effectively inhibited both the hydration (color degradation) and hydrolysis (anthocyanin degradation) of all extracts.

Pearson's correlation analysis indicated a high coefficient of correlation (r = 0.85) between the k values of the color and anthocyanin content. This was an indication that the color and anthocyanin content in all extracts had similar degradation trends. Comparable results were also reported in another study (Marpaung et al., 2017).

As the anthocyanin stability increased because of the elevated amount of sugar added, so too did the stability of the total phenolic compounds. In addition, the coefficient of correlation of k between the total phenolic content and color and between the total phenolic content and the total anthocyanin content were also high (r = 0.91 and r = 0.94, respectively). The strong correlations demonstrated that the color, total anthocyanin content and total phenolic content of all extracts had similar trends of degradation.

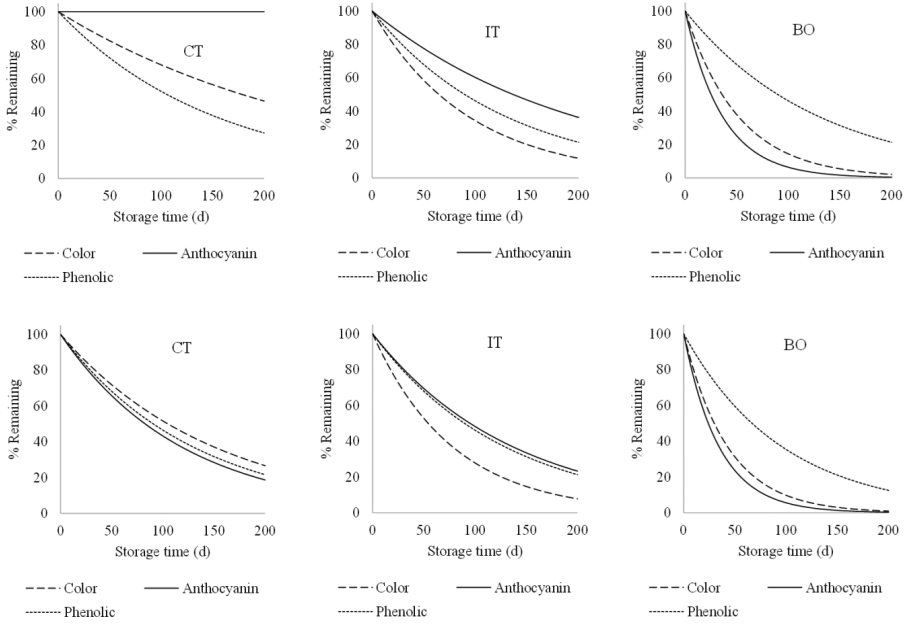

Fig. 2 Models of degradation over storage time for color, anthocyanin content and phenolic content in extracts of *Clitoria ternatea* flowers (CT), *Ipomoea tricolor* flowers (IT) and *Brassica oleracea* leaves (BO) at pH 5 (top row) and pH 6 (bottom row)

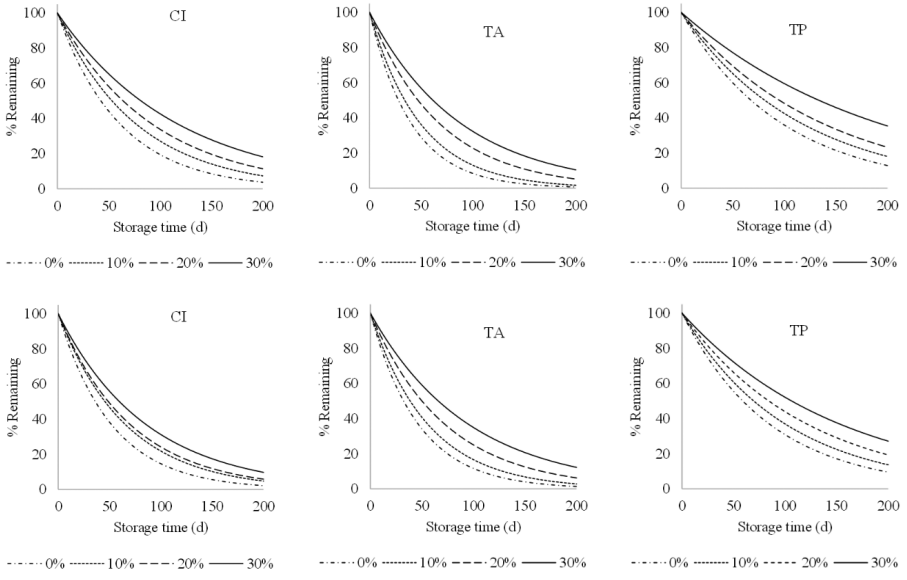

Fig. 3 Models of degradation over storage time for color, anthocyanin content and phenolic content from extract sources (average of three extracts) added with 0%, 10%, 20%, 30% sugar (average of three types of sugar) at pH 5 (top row) and pH 6 (bottom row)

Comparison among extracts

Of interest was that the color, anthocyanin content and total phenolic content stability of the CT extract was higher than the color stability of the IT and BO extracts at both pH levels, at the sugar concentrations studied (Figs. 2 and 3) and also for all sugar types (data not shown). The higher stability was probably due to the chemical structure of the ternatins. The fully opened CT flower has nine types of ternatin that all have an acyl group attached to ring B, for example, ternatin B2, the most abundant anthocyanin in CT, has two acyl groups located at the C3' position and one acyl aromatic group located at the C5' position (Kazuma et al., 2003). It has been reported that the acylated anthocyanins at ring B are more stable than those acylated at ring A or C (Yoshida et al., 2009). The stability of anthocyanins in the CT extract probably contributed to the stability of the total phenolic compounds. This might explain the higher total phenolic stability of the CT extract compared to either of the IT and BO extracts.

The current study has provided significant evidence that the addition of sugar (sucrose, glucose, maltose) improved the anthocyanin stability of extracts from butterfly pea flower, morning glory flower and red cabbage leaves at both pH 5 and pH 6. The butterfly pea extract had a better quality than from the morning glory and red cabbage extracts as sources of natural colorant.

Conflict of Interest

The authors declare no conflict of interest, financial or otherwise.

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